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CO_2 leakage can cause loss of benthic biodiversity in submarine sands

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ABSTRACT

One of the options to mitigate atmospheric CO_2 increase is CO_2 Capture and Storage in sub-seabed geological formations. Since predicting long-term storage security is difficult, different CO_2 leakage scenarios and impacts on marine ecosystems require evaluation. Submarine CO_2 vents may serve as natural analogues and allow studying the effects of CO_2 leakage in a holistic approach. At the study site east of Basiluzzo Islet off Panarea Island (Italy), gas emissions (90–99% CO_2) occur at moderate flows (80–120 $L\,m^{-2}\,h^{-1}$). We investigated the effects of acidified porewater conditions (pH_T range: 5.5–7.7) on the diversity of benthic bacteria and invertebrates by sampling natural sediments in three subsequent years and by performing a transplantation experiment with a duration of one year, respectively. Both multiple years and one year of exposure to acidified porewater conditions reduced the number of benthic bacterial operational taxonomic units and invertebrate species diversity by 30–80%. Reduced biodiversity at the vent sites increased the temporal variability in bacterial and nematode community biomass, abundance and composition. While the release from CO_2 exposure resulted in a full recovery of nematode species diversity within one year, bacterial diversity remained affected. Overall our findings showed that seawater acidification, induced by seafloor CO_2 emissions, was responsible for loss of diversity across different size-classes of benthic organisms, which reduced community stability with potential relapses on ecosystem resilience.

1. Introduction

The combustion of fossil fuels and industrialization are the main causes of the current exceptionally high rates of increase in atmospheric CO_2, which in turn contributes to climate change (Blanco et al., 2014). In the short term, CO_2 Capture and Storage (CCS) in sub-seabed geological formations is considered as the most significant means of reducing net carbon emissions into the atmosphere (Turkenburg, 1997; Bachu and Adams, 2003), while the search for other profitable and renewable energy sources is ongoing. Since it is, however, difficult to predict long-term underground storage security, the inevitable risk of a CO_2 leak from storage reservoirs is one of the arguments raised to dispose CO_2 in aquifers located off-shore instead of land inwards (Turkenburg, 1997; House et al., 2006). Nevertheless, in the advent of a leak, the escaping CO_2 will rapidly dissolve into sediment porewater and into bottom-seawater, reducing the pH and carbonate saturation state and increasing acidity of either (Chen et al., 2005; Millero, 2007). Modeling and experimental studies have shown that the majority of biological impacts from CCS leakage are likely to occur in benthic or epibenthic communities of less mobile organisms, with the magnitude of the impact depending on the duration, the scale and the intensity of the leak, and on the local hydrodynamic regime (Blackford et al., 2009, 2013; Zeppilli et al., 2015).

The majority of CCS leakage impact studies that measured the response of benthic, infaunal or microbial communities applied acidified seawater (pH ≥ 5.6) above sediments in laboratory-based, circulation system experiments (e.g. Dashfield et al., 2008; Ingels et al., 2018; Widdicombe et al., 2009; Schade et al., 2016; Thistle et al., 2006), injected liquefied CO_2 into corrals deposited on the deep-sea floor (porewater pH: 5.4; Carman et al., 2004; Barry et al., 2005) or released CO_2 gas via a borehole at 11 m below the seafloor (porewater pH: 7.5; Widdicombe et al., 2015). Those experiments are very relevant in determining the effects of more or less severe, acute (max. 140 days; Rastelli et al., 2015) CO_2 leakage from CCS sites or injection pipelines. However, they also recognize the inability to predict the impact of more chronic CO_2 leakage or to generalise the results to a more complex

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natural environment, where other environmental and ecological processes can affect the observed responses (Widdicombe et al., 2013). Studies at natural submarine CO_2 vents, where escaping gas is mainly composed of CO_2 and lacks toxic compounds (e.g. sulphide) or temperature increases, may serve to gain this complementary information as they allow to study ecological consequences of relatively long-term (e.g. multi-decadal; Gambi et al., 2003; Jones et al., 2014) exposure to high pCO_2 and low pH porewater conditions in seafloor surface sediments, where porewater pH exhibits a steep decline within the first few millimeters (e.g. pH_T range: 7.7–5.5; Molari et al., 2018). So far, only few studies have used natural gas seeps to investigate the effect of seabed CO_2 leakage on benthic biodiversity (Dias et al., 2010; Pettit et al., 2013; Johnson et al., 2015; Raulf et al., 2015; Hassenrück et al., 2016), focusing only on specific size-class of organisms. Studies at such natural analogues may provide answers to whether species from originally undisturbed natural environments are able to persist and tolerate, and eventually adapt to the highly acidified conditions, or whether they are being replaced by colonizing tolerant species and what consequences this might have for the biodiversity and the role they fulfil within an ecosystem under high biochemical pressure (Molari et al., 2018; Zeppilli et al., 2015).

Molari et al. (2018) found that long-term, high CO_2 conditions at the vents East of Basiluzzo Islet (N of Sicily, SE Tyrrhenian Sea, 14–21 m water depth) led to substantial structural and functional shifts in the bacterial and invertebrate communities, accompanied by a decrease in biomass and abundance, which led to a reduced efficiency in carbon transfer along the food web. Based on a sediment transplantation experiment, Molari et al. (2018) observed that CO_2 leakage impacts on the composition of the benthic communities were already apparent after one year. The recovery of the system after one year of release of exposure to acidified conditions was, however, far from complete. In this study, we focus on the effect of CO_2 leakage on the biodiversity of the vent system at Basiluzzo Islet and explore the relation between biodiversity and temporal stability in structural aspects of the benthos (i.e. community composition, abundance, biomass; data presented in Molari et al., 2018). While we focus on bacteria at the level of operational taxonomic units (OTUs) (i.e. internally transcribed spacer or ITS phylotypes corresponding to binned ARISA peaks), we specifically addressed the most dominant meio- and macrofauna taxa (i.e. nematodes and polychaetes) at species level and tested the following hypotheses:

1) Both one year and at least 3 years of exposure to acidified porewater conditions reduces OTU or species diversity of benthic organisms belonging to different size classes (i.e. microbia, meio- and macrofauna).
2) Benthic bacterial OTU and nematode species diversity do not recover after one year of release of exposure to acidified conditions.

2. Material and methods

2.1. Study site and sampling

The study site is located east of the Basiluzzo Islet, ca. 4 km NE of the Aeolian Island Panarea (Italy), in the SE Tyrrhenian Sea (Fig. 1A). The mainly submarine Aeolian volcanic structure that lies along the NE-orientated faults (Beccaluva et al., 1982; Gabbianelli et al., 1993) is responsible for the visible release of gas from the seabed at several sites around Panarea. Gas emissions at Panarea submarine fumarolic fields have been reported to occur since two thousand years (Italiano and Nuccio, 1991, and references therein; Lucchi et al., 2013, and references therein). Specifically, at the south side of Basiluzzo Islet, shallow (8–13 m water depth) gas emissions have been observed since more than 25 years (Calanchi et al., 1995). This vent area east of Basiluzzo Islet was selected as natural analogue as it fulfills the following criteria: (i) continuous, dispersed degassing of CO_2 through sand causing low pH, (ii) similar oxygen availability and negligible co-emission of toxic substances or microbial energy sources such as sulfide and methane, (iii) no significant temperature anomalies from hydrothermalism (Molari et al., 2018). The sampled locations include two CO_2 vent sites ("CO2-R", N 38°39.749′ E 15°07.123′, 15–17 m water depth; "CO2-G", N 38°39.820′ E 15°07.137′, 21 m water depth) with rather evenly distributed gas leakage (density of 2–3 gas bubble strings per m^2, 120 L m^{-2} h^{-1} and 97–99% CO_2 at CO2-R, 80 L m^{-2} h^{-1} and 90–97% CO_2 at CO2-G) and one reference site ("REF", N 38°39.827′ E 15°07.118′, 14–17 m water depth), with no gas emissions and similar hydrological and sedimentological characteristics as found at the vent sites. CO_2 venting through the sandy sediments resulted in a loss of solid phase carbonate and a decrease in porewater pH by 1–2 units relative to the reference site. A detailed description of the biogeochemistry of the bottom seawater, porewater and, sediments of the selected sampling sites is provided by Molari et al. (2018).

The scuba-diving team collected natural sediment samples in the month June of 2011, 2012 and 2013 for the investigation of the effects of at least 3 years of continuous CO_2-emissions and porewater acidification on the benthos. Macrofauna samples were collected with a push core with an inner diameter of 6.4 cm (only in 2012, n = 5 per site). The upper 5 cm of the sediment was retained and stored unsieved in seawater-buffered formalin (final concentration of 4%). Meiofauna samples were gathered with pre-cut and taped push cores with an inner diameter of 4.7 cm (2011, n = 3 per site) or 5 cm (2012, 2013, n = 3 per site). The sediments were vertically sectioned in 2 cm slices, down to 8 cm depth. All sample sections were preserved in a 4% seawater-buffered formalin solution. Sediment samples for investigating bacterial community composition and diversity, via Automated Ribosomal Intergenic Spacer Analysis (ARISA; Ramette, 2009), were obtained with push cores with an inner diameter of 5.4 cm (2011, n = 2 per site; 2012–2013, n = 3 per site), vertically sectioned in 2 cm slices down to 10 cm depth (n layers = 5), and surficial sediment (0–2 cm) collected with 50 ml plastic tubes (2011, n = 15 per site; 2012–2013, n = 20 per site). Samples were stored frozen at −20 °C. Additionally, 454-Massively Parallel Tag Sequencing (454-MPTS; Sogin et al., 2006) was used for better characterization of the low abundant and rare bacterial taxa in the 0–2 and 4–6 cm sediment layers collected in 2012 (n = 3 per site), as representative for oxic/suboxic and anoxic sediment environments, respectively (Molari et al., 2018).

Additionally, the response of benthic organisms to one year of CO_2-venting or reference conditions was assessed with an *in situ* sediment transplantation experiment. In 2012, sediment samples were transplanted *in situ* within and between REF and CO2-R sites: (i) reimplanted at the same site (within habitat: REF in REF and CO2-R in CO2-R) to control for transplantation effects, and (ii) transplanted to the other habitat type (across habitat: REF in CO2-R and CO2-R in REF) to assess the effect of the different environmental setting (Molari et al., 2018, Fig. 1B). Each transplant was collected after pushing a cylindrical TUBO device into the sediment for 15 cm. Thirty liters of sand were scooped out and transferred into a mesh bag (vinyl-coated fiberglass mosquito net: fiber diameter 280 µm and 1.8 mm × 1.6 mm mesh) covered by an additional plastic bag to protect the sediment from the surrounding seawater during transportation of the bags. At the site of interest, the bags were inserted into an empty TUBO hole (n = 5 per treatment). The TUBO and the plastic bag were removed, and the mesh bags were closed (Molari et al., 2018). Samples for microbial and meiofaunal

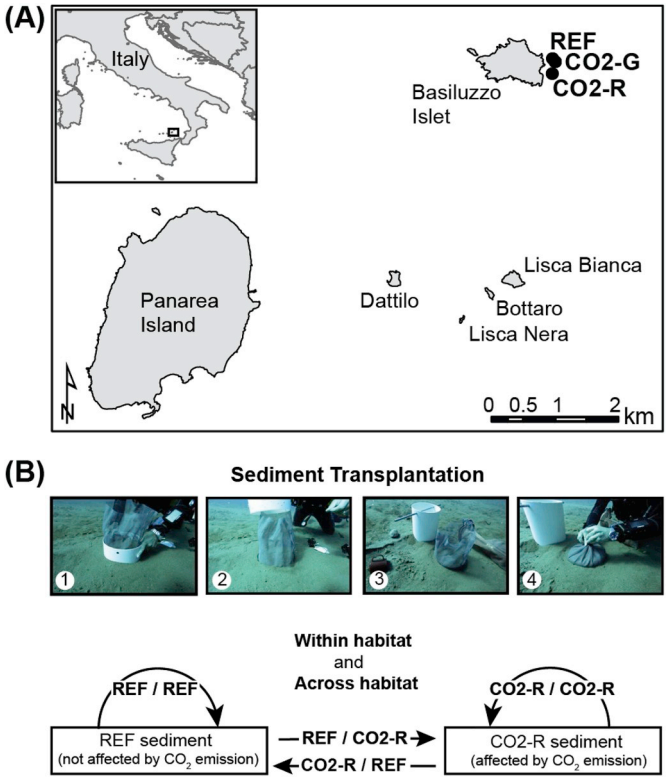

Fig. 1. A) Location of the study area near Basiluzzo Islet (Panarea, Italy), adapted from Guilini et al. (2017b), and **B**) a scheme illustrating the experimental design of the transplantation experiment. The pictures show (1) how a mesh bag (1.8 mm × 1.6 mm mesh) containing 30 L of sediment was inserted in a TUBO device that was first pushed into the sediments (15 cm depth) and emptied, (2–3) the removal of TUBO and the plastic bag, and (4) the closure of the mesh bags.

communities analyses were collected one year later in the same manner as described above. Background sediment samples collected in 2013 from either site were used as control.

2.2. Sample processing

The bacterial community structure was determined with the high-throughput fingerprinting technique ARISA and by applying 454-MPTS on microbial DNA extracts from 1 g of sandy sediment per sediment layer (down to 10 cm for natural and transplanted sediments). A total of 450 OTUs (i.e. ITS phylotypes corresponding to binned ARISA peaks) were detected from 280 sediments samples. 454-MPTS sequencing data of the hypervariable V6 region of the bacterial 16 S rRNA gene were obtained according to the standardized sequencing pipeline applied previously and analyzed with *mothur* standard operating procedure (Version 1.29.2; Schloss et al., 2009). Sequences were clustered into operational taxonomic units at a 3% nucleotide difference (hereafter referred to as $OTU_{0.03}$). The whole dataset comprised a total of 169,490 high-quality bacterial sequences, which were clustered into 9674 $OTUs_{0.03}$. From the whole 454-MPTS subset only OTUs0.03 > 0.1% of total bacterial sequences per sample were used for analysis of dominant classes.

The meiofauna was extracted from the sediment through decantation (5 sessions) and washing on stacked 1-mm and 32-µm mesh sieves. The metazoan organisms were quantified and classified at higher taxon level under a stereoscopic microscope. Fifty to sixty nematodes per sediment layer (down to 8 cm for the natural sediments, down to 4 cm for the transplant experiment) were randomly handpicked with a fine needle, transferred to glycerine (De Grisse I, II and III; Seinhorst, 1959), mounted on glass slides and identified to species (natural sediment) and genus (transplant experiment) level based on original species descriptions which are available on the Nemys website (Guilini et al., 2017a). Nematode biomass (µg dry weight $10\,cm^{-2}$) was determined for all identified nematodes based on Andrassy (1956) and assuming a dry-to-wet-weight ratio of 0.25 (Heip et al., 1985).

The macrofauna was extracted from the sediment through sieving and washing on a 1-mm mesh sieve, followed by handpicking all organisms with a forceps or needle under a binocular microscope. All macrofauna organisms were counted and identified to higher taxon level (down to Class), whilst Polychaeta were further identified to species level based on the World Polychaete Database (Read and Fauchald, 2016) and Gil (2011).

2.3. Data analysis

Alpha diversity was assessed as species richness, exponential of Shannon index and inverse of Simpson index, corresponding to Hill's numbers of order q = 0 (H_0), q = 1 (H_1) and q = 2 (H_2), respectively (Hill, 1973; Chao et al., 2014). The diversity measures spread along Hill's continuum provide us with a more complete understanding of shifts in rare and abundant species and a simplified interpretation of results because units are always in effective number of species (Jost, 2006). These effective species numbers behave as one would intuitively expect when diversity is doubled or halved, while other standard indices of diversity do not (Jost, 2006). For the natural sediments, the overall alpha diversity was visualized in interpolation and extrapolation species diversity curves based on replicates' average of the sum of sequences/individuals per layers. Additionally, for Bacteria, richness estimates (Chao1) were calculated with 100 random re-sampling runs to the smallest number of sequences per sample in the dataset (n = 4346), to account for differences in sequencing depth between samples. Diversity indices were also calculated for bacterial dominant classes, representing together more than 80% of total $OTUs_{0.03}$ at each sites (Molari et al., 2018). For testing the effect of space ("Site" and sediment "Layer") and time ("Year") on variations observed in bacterial, nematode and polychaete alpha diversity from natural sediments, multifactorial ANOVA analyses were performed based on a three factor design with Layer nested in Site. The nested design was chosen to take into account the site-specific environmental gradients due to presence/absences of acidified porewater effluxes at CO2-venting sites and reference, respectively (for more details see Molari et al., 2018). In order to account the effect due to transition between aerobic and anaerobic environments, the diversity indices of single layer were grouped in oxic/suboxic (i.e. 0–2 cm) and anoxic (below 2 cm) layers. Moreover, for bacteria we had more observations for 0–2 cm layer (n = 15–20) than for other layers (n = 3), thus grouping together layers below 2 cm evened sampling effort. In order to account for the dependency of observation from the same sediment core, a mixed effects model with "Sediment Core" as random factor was carried out for Nematode data. Three-way ANOVA was applied to Bacteria and one-way ANOVA to Polychaete data due to high contribution of independent observations for layer 0–2 cm and one observation per core, respectively. To test the effect of short-term exposure to acidified porewater conditions on alpha diversity of bacteria and nematodes, a two-way split-spot ANOVA was performed with the factors "Treatment" (within habitat transplants and across habitats transplants) and sediment "Layer" (nested in

"Treatment"), and "Sediment Core" as random factor. Transplants pairwise comparison was carried out using non-parametric Kruskal-Wallis tests with Benjamini–Hochberg (BH) adjusted P-values.

Temporal stability (S), which is an index of the stability of a community or population over time (Tilman, 1999; Lehman and Tilman, 2000; Tilman et al., 2006), was considered for bacterial and nematode density, biomass and community composition (data which are presented in Molari et al., 2018). S was estimated as the ratio between the temporal mean (μ) of community-level density, biomass and Bray-Curtis similarity values for species composition and their standard deviation (σ), per sampling site. Larger S values indicate higher temporal stability. The relationship between temporal stability and biodiversity could only be visually stated since three sample sites did not allow performing simple linear regressions.

Beta diversity (i.e. OTUs/species turnover) was calculated as the Jaccard dissimilarity distance on presence/absence data for each of the bacteria, nematode and polychaete datasets. Turnover of OTUs or species were quantified between the layers and between the years at each site for Bacteria (ARISA) and nematodes, and between sites for bacteria (ARISA and 454-MPTS), nematodes and polychaetes. The Jaccard dissimilarity distance was used to calculate beta similarity (i.e. the number of shared OTUs/species), and similarity percentage breakdown procedure (SIMPER; Clarke, 1993) was carried out to assess the average percentage of shared OTUs/species within and between the groups (sites and layers per each year, and years per each site) for Bacteria (ARISA and 454-MPTS) and Nematoda datasets. One- or two-way ANOVA, after verifying that the assumptions of normality and homoscedasticity were met, was applied to test significant differences between average of shared OTUs/species. Following significant ANOVA between the investigated sites and/or years, Tukey post-hoc comparison tests were applied. In case the assumptions for ANOVA were not met, Kruskal-Wallis tests and post-hoc Dunn-tests were performed instead, with BH-adjusted P-values.

Multivariate Redundancy Analyses (RDA) were performed to investigate which environmental variable or set of variables (Table A0.1) could best explain the patterns in bacterial and nematode diversity (Hill's numbers) observed with depth in the sediment (0–10 cm and 0–8 cm, respectively) and in the oxic/suboxic layer (0–2 cm) for the years 2012 and 2013. Prior to the analyses, the explanatory environmental variables were standardized (i.e. Z-scored) and assessed for collinearity based on the variance inflation factor (VIF). The variables pH, chlorophyll *a* (Chl-a), total organic carbon (TOC), median grain size (MGS), silicate, and dissolved inorganic carbon (DIC) were retained based on a VIF < 3, indicating no or little collinearity among these variables. Additionally, variation partitioning (VP) analysis was used with multiple partial RDAs to assess the explanatory power of each significant explanatory variable and proportion of variance explained by variables multicollinearity. All statistical analyses were performed in R (v. 3.3.1) (R Development Core Team, 2014; https://www.R-project.org) using packages iNEXT (Hsieh et al., 2016), vegan (Oksanen et al., 2015), usdm (Naimi et al., 2014), lme4 (Bates et al., 2015), and ggplot2 (Wickham, 2009).

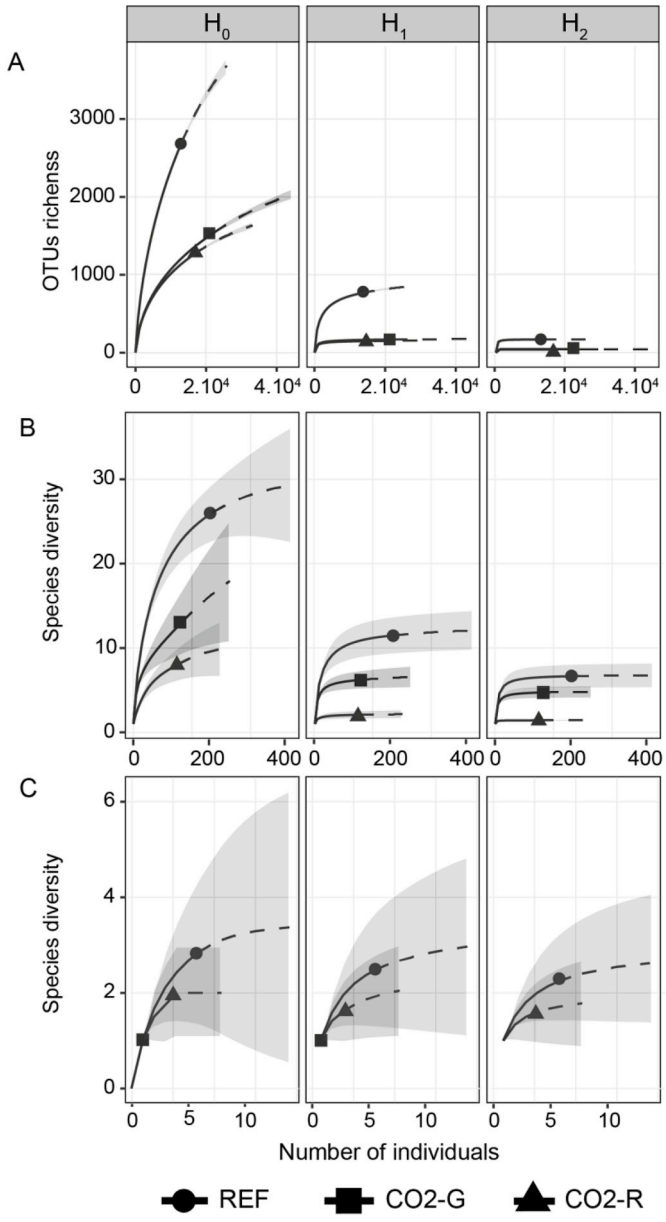

Fig. 2. Rarefaction curves based on Hill numbers (q = 0, 1 and 2) for **A**) numbers of sequences of Bacteria (454-MPTS, n = 3, 0–2 cm and 4–6 cm), **B**) abundance of Nematoda (n = 3, 0–8 cm), and **C**) abundance of Polychaeta (n = 5, 0–5 cm) for samples collected in 2012. Interpolation (full), extrapolation (dashed) lines and their 95% confidence intervals (shaded area) are indicated. The rarefaction curves for the nematode community sampled in 2011 and 2013 are very similar to the curves for 2012 and are shown in Fig. A1.

3. Results

The rarefaction curves indicated that bacterial (OTUs), nematode (species) and polychaete (species) richness in the natural sediments was well captured for the abundant taxa in all three study sites, while diversity of the less abundant taxa and rare biosphere remained largely underestimated (Fig. 2, Fig. A1 and A2). REF contributed the largest fraction of absolute singletons ($OTUs_{0.03}$ with single-sequence; SSO_{abs}) and unique $OTUs_{0.03}$, followed by CO2-G and then by CO2-R (Table A.2). Hill numbers (Fig. 3 and A.3) showed a general reduction in diversity at the CO_2-venting sites compared to the reference site (ANOVA, $p < 0.001$, redundancy ≥ 63.1%; Table A.3), with no more than 1.5% and 21% of the variance explained by "Year" (2011–2013) and sediment "Layer" (0–2 cm and 2–10 cm for bacteria, 0–2 cm and 2–8 cm for nematodes), respectively, for both bacteria and nematodes. Bacterial diversity (OTUs) was generally the lowest at CO2-R with almost all

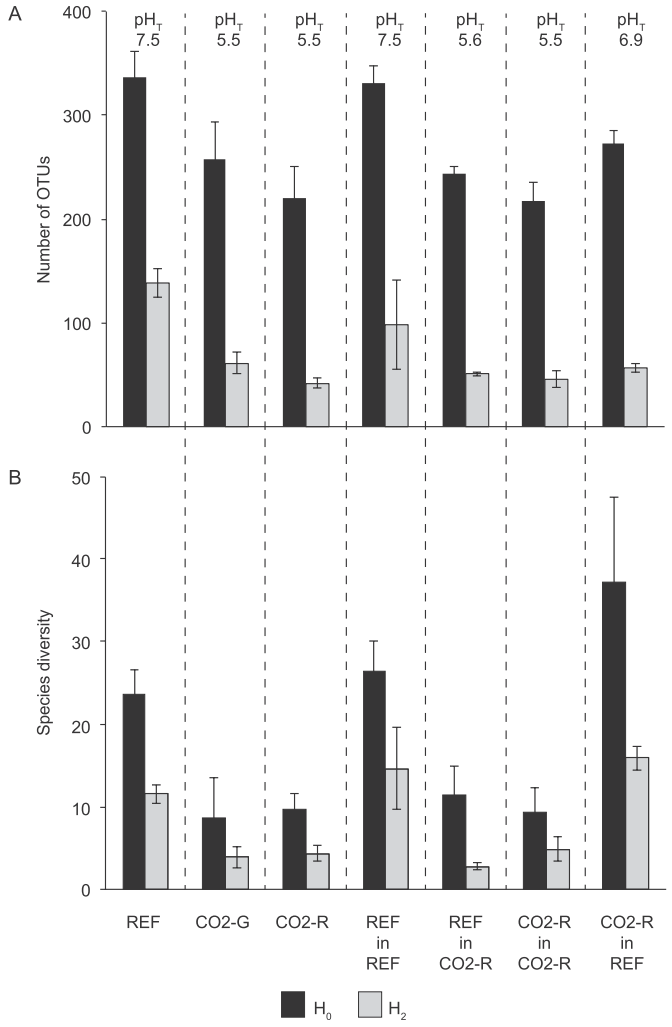

Fig. 3. Sediment integrated alpha diversity expressed in Hill's numbers (q = 0, 2) for **A**) Bacterial OTUs (0–10 cm, ARISA, undisturbed sediments 2011–2013, n = 9, transplanted sediments 2013, n = 3) and **B**) Nematoda genera in the natural sediments at REF, CO2-G and CO2-R (0–4 cm, n = 9), and in the transplanted sediments within and across REF and CO2-R (0–4 cm, n = 3). The provided pH_T values are the averages in the 0–2 cm sediment layer.

dominant classes showing a decrease of richness and evenness at the seep sites (Fig. A.4). Diversity of polychaete species was equally low at both vent sites for all indices (ANOVA pairwise test, $p \geq 0.72$) while nematode species diversity was equally low at both vent sites for H_0 (ANOVA pairwise test, $p = 0.35$) and lower at CO2-R compared to CO2-G for H_1 and H_2 (ANOVA pairwise test, $p \leq 0.030$). Redundancy analyses and variation partitioning revealed that alpha diversity was mainly determined by pH (29–56%; Table 1). Other biogeochemical variables explained no more than 11% individually (Table 1, Fig. A.5).

Transplantations experiments had a significant impact on bacterial and nematode alfa-diversity (Table A.4 and Fig. 3). Internal-site transplanting sediments had a negligible disturbance effect on the nematodes, since alpha diversity in "within habitat" transplants did not differ from the natural samples (Fig. 3; REF *vs* REF/REF: H_0 Kruskal-Wallis, $\chi^2 = 2.44$, adjusted $P > 0.2$, H_2 Kruskal-Wallis, $\chi^2 = 1.26$, adjusted $P > 0.4$; CO2-R *vs* CO2-R/CO2-R: H_0 Kruskal-Wallis, $\chi^2 = 0.03$, adjusted $P > 0.18$, H_2 Kruskal-Wallis, $\chi^2 = 0.41$, adjusted $P > 0.5$), and just a minor impact on bacterial diversity (Fig. 3; REF *vs* REF/REF: H_0 Kruskal-Wallis, $\chi^2 = 0.69$, BH-adjusted $P > 0.4$, H_2 Kruskal-Wallis, $\chi^2 = 5.69$, adjusted $P < 0.05$; CO2-R *vs* CO2-R/CO2-R: H_0 Kruskal-Wallis, $\chi^2 = 7.32$, adjusted $P < 0.01$, H_2 Kruskal-Wallis, $\chi^2 = 3.88$, adjusted $P < 0.05$). Reference sediments transplanted at the vent site showed a reduction in bacterial and nematode diversity (REF *vs* REF/CO2-R: bacteria H_0 Kruskal-Wallis, $\chi^2 = 21.79$, adjusted $P < 0.0001$, bacteria H_2 Kruskal-Wallis, $\chi^2 = 21.77$, adjusted $P < 0.0001$; Nematode H_0 Kruskal-Wallis, $\chi^2 = 8.46$, adjusted $P < 0.05$, Nematode H_2 Kruskal-Wallis, $\chi^2 = 8.31$, adjusted $P < 0.05$), suggesting a significant impact of one-year exposure to high pCO_2/low pH conditions (Fig. 3). In sediments transplanted from the vent site CO2-R to REF only nematode richness was higher compared to the natural undisturbed REF sediments, whereas bacterial diversity was still significantly reduced (Fig. 3; REF *vs* CO2-R/REF: bacteria H_0 Kruskal-Wallis, $\chi^2 = 18.63$, adjusted $P < 0.0001$, bacteria H_2 Kruskal-Wallis, $\chi^2 = 21.00$, adjusted $P < 0.0001$; Nematode H_0 Kruskal-Wallis, $\chi^2 = 6.38$, adjusted $P < 0.05$, Nematode H_2 Kruskal-Wallis, $\chi^2 = 5.77$, adjusted $P < 0.05$).

Interestingly, with the decreasing of species diversity (Hill's numbers) at vent sites, there was a clear trend of decreasing of temporal stability (S) in bacterial and nematode density and community composition, as well as in nematode biomass (Fig. 4).

The number of shared OTUs/species in the natural sediments was generally lower between the reference and CO_2-venting sites than between the two vent sites (Table 2; Bacteria (ARISA): ANOVA, F value = 8.19, adjusted $P < 0.05$; Bacteria (454-MPTS): Kruskal-Wallis, $\chi^2 = 12.79$, adjusted $P < 0.001$; Nematoda: Kruskal-Wallis, $\chi^2 = 79.78$, adjusted $P < 0.0001$; Polychaeta: Kruskal-Wallis, $\chi^2 = 22.6$, adjusted $P < 0.001$). Beta similarity was different among size-class organisms here investigated, as REF shared 9–10% of nematode species and 0–3% of polychaete species, while 82–85% (ARISA) and 16–20% (454-MPTS) of bacteria OTUs with the vents. The number OTUs and species shared between the top sediment layer (0–2 cm) and underlying layers decreased with sediment depth at REF and CO_2 vents in all years (Table 3). Even, the beta similariy between top and deepest layers of the sediment were higher at REF than at vent sites both for Bacteria and nematodes (Bacteria (ARISA): ANOVA, F value = 25.91, adjusted $P < 0.001$; nematodes: Kruskal-Wallis, $\chi^2 = 25.36$, adjusted $P < 0.001$). Temporal variation in bacterial and nematode community composition was lower at REF than at CO_2-venting sites (Table 2; Bacteria (ARISA): ANOVA, F value = 18.02, adjusted $P < 0.01$; Nematoda: ANOVA, F value = 128.09, adjusted $P < 0.0001$).

4. Discussion

4.1. CO_2 leakage affects bacterial diversity

The results from few previous studies investigating effects of acidified porewater on bacterial alpha diversity in soft sediments at CO_2 venting sites showed contradictory responses (Yanagawa et al., 2013; Kerfahi et al., 2014; Raulf et al., 2015; Hassenrück et al., 2016), likely as a result of the inherent high heterogeneity of environmental gradients at natural vents (German and Seyfried, 2014). Where no pure CO_2 release occurs, fluid and gas emissions of reduced elements provide potential energy sources for specialized microorganisms which leads to a shift in composition of the benthic microbial communities, favoring specific functional groups (i.e. chemolithotrophs; Price et al., 2015) and consortia (e.g. anaerobic methanotrophs; Boetius et al., 2000) that may mask the microbial responses to CO_2. In the CO_2-venting sites here investigated, Molari et al. (2018) did not find presence of hydrothermal endemism, and we observed a lower beta diversity compared to those

Table 1
Results of variation partitioning and partial redundancy analyses illustrating which predictor variables explain the variance in Bacteria (ARISA) and Nematoda diversity (Hills numbers) for 2012 and 2013, considering the entire sediment profile (all layers) and restricted to the upper 2 cm of the sediment. Proportion of variance explained (%) and p values (ANOVA) are reported for significant variables, as identified by RDA (single source). Proportion of variance explained by variables multicollinearity is also reported, as identified by variation portioning analysis (multiple sources). RDA and partial RDA details are reported in Table A0.5.

Habitat	Undisturbed sediments				Undisturbed sediments			
Assemblage	Bacteria				Nematoda			
Layer	0–10 cm		0–2 cm		0–8 cm		0–2 cm	
observations	n = 90		n = 18		n = 72		n = 18	
	Variance (%)	P-value	Variance (%)	P-value	Variance (%)	P-value	Variance (%)	P-value
Single source								
pH	40	0.001	55	0.001	56	0.001	38	0.001
MGS	< 1	ns	< 1	nt	0	nt	11	0.005
Silicate	2	0.003	6	0.007	0	nt	5	0.047
Chl-a	0	nt	0	nt	1	0.022	0	nt
Multiple sources								
pH + MGS	6		1		0		12	
pH + Silicate	10		18		0		0	
pH + MGS + Silicate	20		12		0		16	
pH + Chl-a	0		0		17		0	
Unexplained	20		8		25		18	

MGS = median grain size; Chl-a = chlorophyll *a*; ns = not significant; nt = not tested.

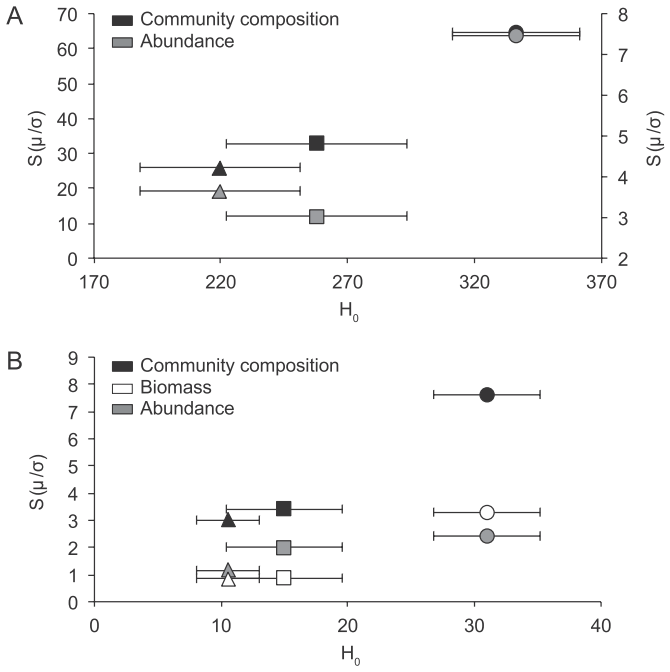

Fig. 4. Relationships between sediment integrated OTUs and species richness (H_0) and temporal stability (S) over 3 years across all study sites (n = 9). **A)** Bacterial temporal stability based on community composition (left Y-axis) and abundance (right Y-axis), and **B)** nematode biomass, density and community composition based on species level, across all study sites (circle: REF, square: CO2-G, triangle: CO2-R).

reported for other ocean acidification natural analogues (Raulf et al., 2015; Hassenrück et al., 2016) and typically described for seeps and hydrothermal vents (Ristova et al., 2015; Raulf et al., 2015). Besides, the temporal variation in community composition was comparable with that observed in other coastal sandy sediments (Böer et al., 2009; Gobet et al., 2012). Direct comparison of beta diversity values between studies could be biased by application of different sequencing techniques (e.g. different sequencing depth, different primers) and methods for beta diversity estimation. However, here, our results were compared with previous studies applying the same methods to investigate microbial diversity (i.e. ARISA and/or 454-MPTS) and to assess beta diversity (i.e. Jaccard dissimilarity distance). Both long- (at least three years) and short-term (one year) exposure to CO_2 emissions resulted in a decrease

Table 2
Beta similarity between sites for three years and between years within study sites, presented as the average percentage (± standard deviation) of shared OTUs/species derived from Jaccard dissimilarity index based on presence/absence data for bacterial OTUs (ARISA, n = 3; 454-MPTS, n = 9), nematode (n = 3) and polychaete (n = 5) species. 454-MPTS was only applied on samples collected in 2012, therefore replicates' pairwise comparisons were used to test differences in beta similarity between site, and beta similarity between years is not available (na).

	Shared bacterial OTUs (%)				Shared nematode species		Shared polychaete species	
	ARISA		454-MPTS		(%)		(%)	
Between sites								
REF vs CO2-R	68	± 7	16	± 1	9	± 7	3	± 7
REF vs CO2-G	73	± 3	20	± 2	10	± 7	0	± 0
CO2-R vs CO2-G	82	± 2	32	± 2	20	± 8	33	± 32
Between years								
REF	88	± 1	na	na	47	± 6	na	na
CO2-R	76	± 3	na	na	29	± 7	na	na
CO2-G	82	± 3	na	na	18	± 6	na	na

Table 3
Beta similarity between sediment depth layers at each study site, presented as the average percentage (± standard deviation) of shared OTUs/species derived from Jaccard dissimilarity index based on presence/absence data of three years (2011–2012) for bacterial OTUs (ARISA) and nematode species (n = 3); *a* is the percentage of bacterial OTUs or nematode species shared with 0–2 cm, *b* is the percentage of bacterial OTUs or nematode species shared with previous layer.

		Shared bacterial OTUs (%)				Shared nematode species (%)			
		a		*b*		*a*		*b*	
REF	0–2 cm	–	–	–	–	–	–	-	–
	2–4 cm	82	± 4	–	–	41	± 10	–	–
	4–6 cm	79	± 4	83	± 4	34	± 9	40	± 10
	6–8 cm	76	± 3	81	± 4	27	± 12	31	± 10
	8–10 cm	76	± 4	84	± 3	–	–	–	–
CO2-G	0–2 cm	–	–	–	–	–	–	–	–
	2–4 cm	73	± 7	–	–	41	± 17	–	–
	4–6 cm	71	± 3	81	± 1	28	± 14	32	± 15
	6–8 cm	67	± 4	77	± 4	18	± 13	19	± 21
	8–10 cm	67	± 7	78	± 5	–	–	–	–
CO2-R	0–2 cm	–	–	–	–	–	–	–	–
	2–4 cm	72	± 7	–	–	26	± 13	–	–
	4–6 cm	68	± 8	80	± 5	26	± 16	28	± 13
	6–8 cm	62	± 5	78	± 8	21	± 14	23	± 14
	8–10 cm	60	± 8	78	± 11	–	–	–	–

of microbial diversity in benthic microbial assemblages at Basiluzzo. Remarkably, the decrease in richness and evenness was observed within almost all dominant bacterial classes, even those favored at CO_2 vent sites (Molari et al., 2018). Together, these findings suggest a stable and univocal response of dominant bacterial functional groups to CO_2 emissions here investigated. Molari et al. (2018) showed that alongside the lowering of pH, the CO_2 emissions have an indirect effect on productivity (i.e. stimulating microphytobenthos) and sand mineralogy (i.e. dissolution of carbonate fraction). The trophic status and sediment composition are known to be factors shaping microbial communities (e.g. Schöttner et al., 2011; Bienhold et al., 2012). However, our results showed that bacterial diversity was highly related with pH rather than with others environmental factors, suggesting that at venting sites CO_2 emissions led to the selection for those bacteria physiologically tolerant to acid stress.

Neutralophilic bacteria, growing at pH range of 5–9, can use different mechanisms for pH homeostasis (Booth, 1985; Slonczewski et al., 2009). Specifically for respiratory bacteria, which dominated microbial communities at our study sites, acid stress is met by direct active efflux of protons by redox potential-driven pumps, light-driven pumps or bond energy-driven pumps (Krulwich et al., 2011). Recently, a 9-days mesocosm experiment showed that a pH reduction of 0.2 units stimulated expression of different pH homeostasis genes in marine heterotrophic bacterioplankton (Bunse et al., 2016). These stimulated mechanisms to export protons across the cell membrane are energy demanding, which implies that the physiological acclimation of bacteria to seawater acidification may increase energy demand for cell maintenance, potentially resulting in a reduction of growth efficiency. Thus, under exposure to constant and moderate flows of acidified seawater, and depending on the extent of acidification, we can expect that an increase of metabolic cost could select for those bacteria with more versatile metabolism (e.g. resource exploitation, regulation of growth efficiency), with consequences for diversity and carbon cycling. This is exactly what we have observed at acidified Basiluzzo sites, where the unchanged bacterial abundance was accompanied with a loss of diversity and an increase of organic carbon remineralization (Molari et al., 2018). Future studies need to be carried out to look closely at the effects of seawater acidification on microbial physiology and energy allocation for elucidating the consequence on carbon and energy fluxes in the microbial food web. Nevertheless, for the fist time transplantation experiments and three years of field observations provide robust evidence that seawater acidification, induced by seafloor CO_2 emissions, can be responsible for the loss of bacterial diversity.

4.2. CO_2 leakage affects invertebrate diversity

The results of this study demonstrate that at least 2 years of exposure to diffuse CO_2 leakage reduced polychaete species diversity. These findings complement the results from laboratory and field experiments investigating more or less severe, acute CCS leakage impacts on benthic macrofaunal diversity (Christen et al., 2012; Hale et al., 2011; Ingels et al., 2012; Widdicombe et al., 2009, 2015). The threshold at which impacts were observed on infaunal macro-invertebrate diversity in laboratory experiments that mimic a CO_2 rich plume, was identified at seawater pH levels below 7, but only after five weeks of continuous exposure (Widdicombe et al., 2015). An experimental subseabed release of CO_2 caused a reduction in macrobenthic diversity within a few days, although porewater pH did not drop below 7.5 (Widdicombe et al., 2015). In both scenarios, impact thresholds are likely determined by natural processes, such as carbonate buffering and permeability, which influence the carbon chemistry of the sediment (Widdicombe et al., 2015). Moreover, the vulnerabilities to high pCO_2/low pH conditions differ between and within phyla (Christen et al., 2012 and references therein). The reduction in biodiversity in these relatively short-term CO_2 leakage simulations result from the loss of species that hamper the ability to regulate the acid-base balance of internal fluids to maintain a number of key pH sensitive physiological processes (Kroeker et al., 2013) or in case of calcifying organisms, fail to maintain important physiological processes (e.g. growth, reproduction, immune function) as a result of an energetically more demanding calcification process under reduced calcite or aragonite saturation states (Pörtner, 2008; Wood et al., 2008). In general, annelids were classified among the taxa most resilient to highly elevated pCO_2/low pH (Christen et al., 2012; Hale et al., 2011). The relatively long-term

perspective and natural environment in our study allows to consider whether the vacant niches of species that disappear on the short term are being occupied by colonizing tolerant species and what consequences this might have for the biodiversity. The low number of shared polychaete species between the reference and vent sites and the severely reduced species diversity at the vent site leaves no doubt that polychaete communities cannot cope with diffuse CO_2 leakage over a period of at least several years. This reduction in species diversity coincides with a reduction in functional diversity (i.e. feeding modes), density and biomass (Molari et al., 2018) and therefore warrants for a reduction in the optimal functioning of the benthic ecosystem.

Nematodes, generally the numerically dominant and species-rich members of the meiobenthos, have most often been the focus of meiobenthic response measures to acute seawater acidification (Ingels et al., 2012, 2018; Kurihara et al., 2007). This is the first study, however, that indicates that one to at least three years of exposure to acidified porewater conditions (pH_T minimum: 5.5) in a natural environment severely reduces nematode species diversity. Earlier acute acidification exposure experiments testing the effect of seawater pH ≥ 6 over a maximum of 12 weeks on the meiobenthos found no negative effects on nematode abundance, community composition and diversity (Takeuchi et al., 1997; Dashfield et al., 2008; Ingels et al., 2018; Schade et al., 2016). Merely once an increase in nematode abundances occurred, which was attributed to the reduction of ecological constraints (predation, competition) resulting from a decrease in macrofaunal abundance (Hale et al., 2011). Only when seawater acidity was decreased below what appears to be a threshold of 6 pH units for a maximum of 20 weeks, nematode community structure changed and species diversity decreased (Widdicombe et al., 2009), abundance decreased (Takeuchi et al., 1997; Barry et al., 2004; Ishida et al., 2005), or high mortality was suggested based on changed morphometrics (Fleeger et al., 2010). The nematodes' impermeable proteinaceaous cuticle is suggested to buffer them relatively well to short-term natural fluctuations of porewater pH in the upper few centimeters of sediments (typical pH range of 6.5–8.2; Brusca and Brusca, 1990; Widdicombe et al., 2011). Persistently high CO_2 conditions probably resulted in extracellular acidosis, a condition which might exert lethal physiological stresses on nematodes (Barry et al., 2004; Widdicombe et al., 2009). The highly distinct and species-poor nematode communities found at the vents at Basiluzzo Islet, however, also demonstrate that there are nematode species which potentially adapted and/or exhibit tolerance to extreme and chronic high pCO_2/low pH conditions, with few opportunistic species demonstrating strong competitive advantages in exploiting these harsh environments (e.g. *Microlaimus compridus*, *Microlaimus honestus*, *Oncholaimus campylocercoides*, and *Daptonema Microspiculum*; Molari et al., 2018). At the same time, these species-poor vent communities seem prone to a relatively high temporal variability in different structural community characteristics (i.e. community composition, abundance, biomass), which suggests reduced resilience to additional environmental perturbations. These results emphasize the importance of investigating synergistic impacts of environmental factors on multi-species assemblages to enable more informative predictions (Jones et al., 2014; Mevenkamp et al., 2018).

Similar susceptible changes to CO_2 exposure were also observed for other meiobenthic groups (Thistle et al., 2005). For example, for many species of harpaticoid copepods, alterations in CO_2 concentrations resulted in general mortality rates of around 80% of the total community (Thistle et al., 2006), but it also indicated that some species are less susceptible than others to changes in the environment (Thistle et al., 2006, 2007).

In general, the meiobenthos is less affected by environmental changes compared to the macrobenthos (Ingels et al., 2012; Zeppilli et al., 2015). Some species can even be favored by extreme environmental conditions. Within the meiobenthos, copepods are considered particularly susceptible to stress in comparison with nematodes, suggesting the high versatility and different responses of this size class in predicting major global changes by identifying sensitive and tolerant species (Zeppilli et al., 2015).

4.3. Recovery of benthic diversity after CO_2 leakage has ceased

Transplanting sediments from the CO_2 vent site to the reference site allowed evaluating the recovery potential of high pCO_2/low pH-impacted nematode communities. Our results show that nematode species diversity could re-establish within one year, while Molari et al. (2018) additionally demonstrates a recovery of the nematode community composition, though with reduced overall population densities. These findings are in agreement with the results presented by Widdicombe et al. (2015), where the macrofauna community composition had recovered from the impact of a subseabed CO_2 injection 18 days after the leakage was stopped. Neither the bacterial community composition (Molari et al., 2018), nor the diversity of bacteria recovered within one year, suggesting that colonization time exceeded one year. However, as mentioned above, the CO_2 leakage promoted dissolution of carbonate sands, an effect that was still recorded in the sediments transplanted to the reference site (Molari et al., 2018). Without carbonate sands the sediment lost its buffering capacity, resulting in porewater with a pH value 0.4–0.7 units lower than what is typically found in undisturbed sediments. Thus, we cannot exclude that bacterial diversity may be affected by variations of pH values even smaller than those observed at CO_2-venting sites.

Although both experiments have shown that the chemical effects of CO_2 leakage on benthic invertebrate diversity are not long lasting, we need to consider that both study areas were spatially relatively small (35–200 m^2). The time needed for recovery from CO_2 leakage events will eventually depend on the area of the impacted site, the flux of CO_2 and the potential physical disruption of the sediments, the species dispersal capabilities, their rate of recruitment, and the distance between the disturbed site and the unaffected source populations.

5. Conclusions

In summary, this study proved that porewater acidification, induced by seafloor CO_2 emissions, was responsible for reducing 30–80% species diversity of dominant benthic organisms across size-classes (Bacteria, Nematoda and Polychaeta). With the transplantation experiment we provided evidence that the impact on microbe and nematode diversity was already apparent after one year of exposure to CO_2 emission. Additionally, the transplantation experiment showed that recovery of an impacted area relatively small in size was fairly quick (i.e. within one year), at least for nematodes. The lack of recovery of bacterial communities pointed out that effects of CO_2 leakage on sediment properties may last for a long time after the disturbance is ceased, with consequences on benthic biodiversity that may vary according to the size and ecology of organisms (e.g. dispersion, sessile/motile) and to the scale of the impacted area.

High microbial and nematode species richness at the reference site revealed more stable communities over time, while loss of species at the vent sites resulted in higher temporal variability in community characteristics, suggesting reduced resilience to additional environmental perturbations at impacted locations. These results contribute to the growing body of evidence that highlights the importance of biodiversity for ecosystem stability.

Data accessibility

Data can be accessed through the PANGAEA database (https://www.pangaea.de). Biodiversity is calculated based on data that are deposited in the following datasets: #830368, #846151, #846154, #846155, #846597, #873500. During the review process, the reviewers can access these data and also the supporting data from Molari et al. (2018) via https://doi.pangaea.de/10.1594/PANGAEA.871453.

Authors' contributions

K.G. processed the macro- and meiofauna samples, identified the nematodes to species level, analysed the faunal data, and wrote the manuscript; M.M. processed the bacterial samples, analysed the bacterial data, and wrote the manuscript; L.L. processed the meiofauna samples and wrote the manuscript; A.V., A.R. and K.G. designed the study. All authors revised the article critically and gave final approval of the version to be published.

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Appendices

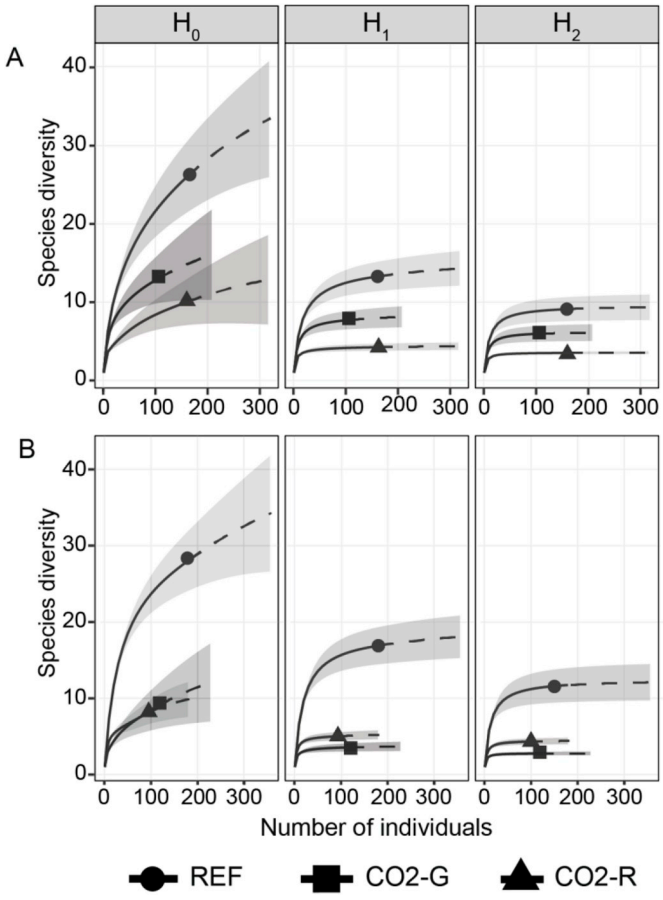

Fig. A.1. Rarefaction curves based on Hill numbers (q = 0, 1 and 2) for **A**) abundance of Nematoda for samples collected in 2011 (n = 3) and **B**) abundance of Nematoda for samples collected in 2013 (n = 3). Interpolation (full), extrapolation (dashed) lines and their 95% confidence intervals (shaded area) are indicated.

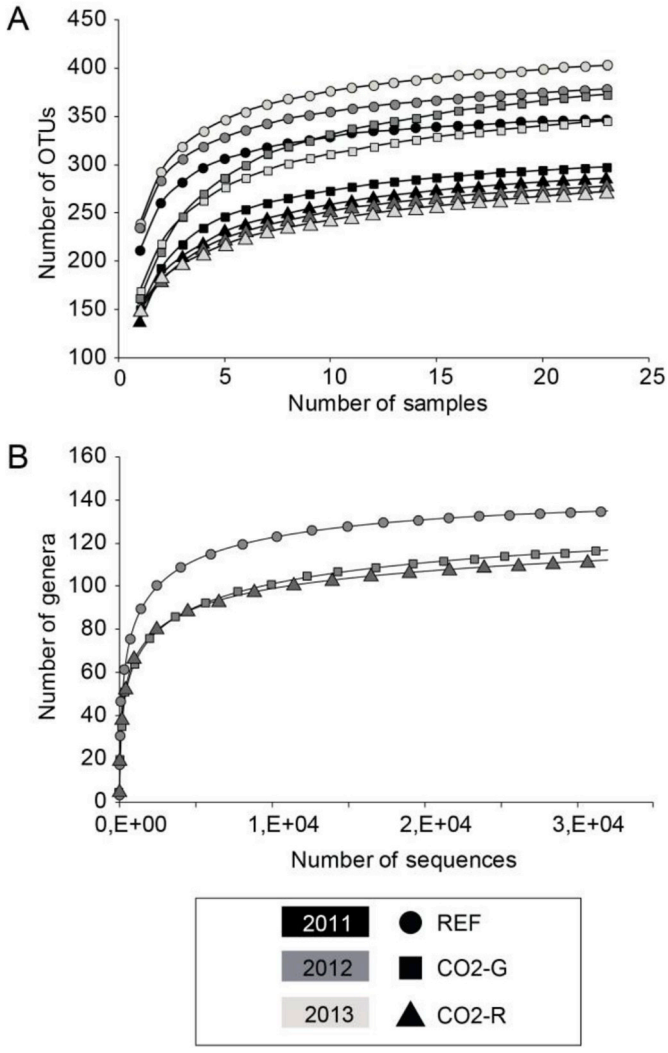

Fig. A.2. Rarefaction curves calculated from **A**) numbers of bacterial sequences based on the ARISA (2011–2013) and **B**) numbers of genera based on 454-MPTS (2012; n = 3).

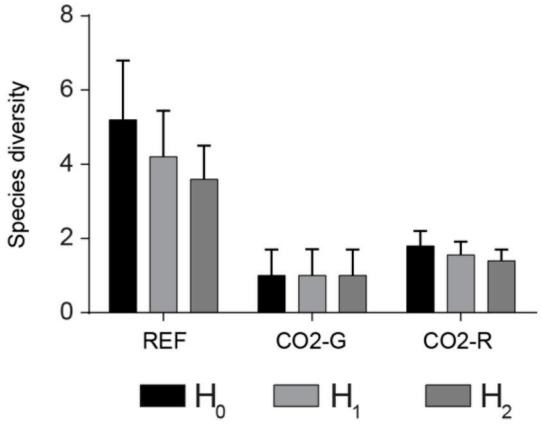

Fig. A.3. Alpha diversity of Polychaeta species (n = 5) expressed in Hill's numbers (H_0, H_1, H_2) for each study site (REF, CO2-G, CO2-R).

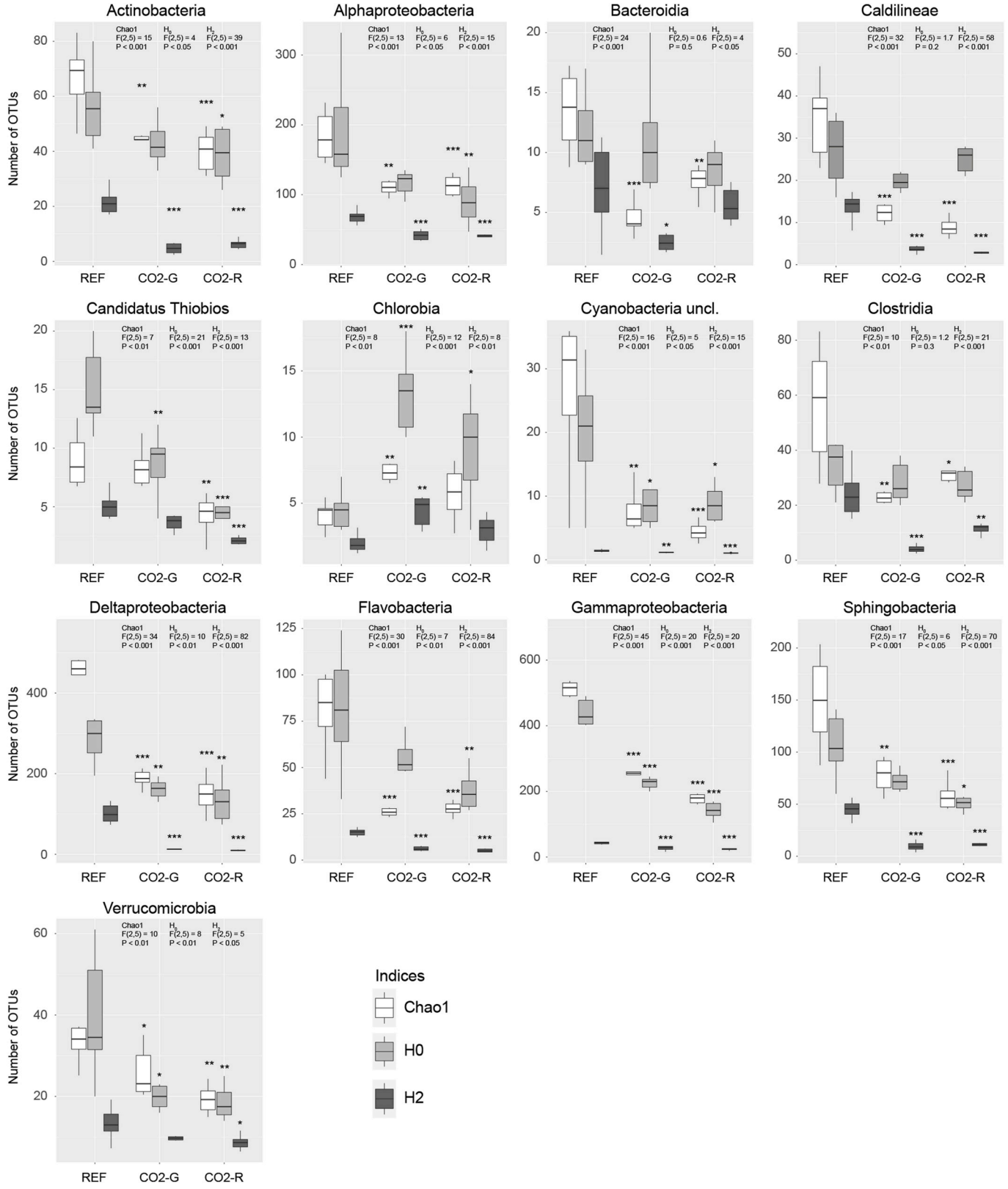

Fig. A.4. Alpha Diversity as described by estimated richness (Chao1) and Hills numbers (H0 and H2) for dominat bacterial classes, based on 454-MPTS data ($OTUs_{0.003}$ > 0.1%; n = 6; 0–2 cm 4–6 cm layer were combined). Significant difference between the vent sites and the REF are indicated with an asterisk according to pairwise comparison adjusted P values (* < 0.05, ** < 0.01, *** < 0.001). In the plot statistic *F-ratio* (F), with subscript numbers reporting the degrees of freedom between groups and within groups, respectively, and probability level (P) for total ANOVA model are also reported. Chao1 was calucalted based on repeated random subsampling of the 454-MPTS data sets for each class: Actinobacteria = 175 sequences; Alphaproteobacteria = 132 sequences; Bacteroidia = 11 sequences; Caldilineae = 32 sequences; Candidatus Thiobios = 19 sequences; Chlorobia = 15 sequences; Cyanobacteria unclassified (uncl.) = 122 sequences; Clostridia = 32 sequences; Deltaproteobacteria = 470 sequences; Flavobacteria = 185 sequences; Gammaproteobacteria = 860 sequences; Sphingobacteria = 187 sequences;

Verrucomicrobia = 39 sequences.

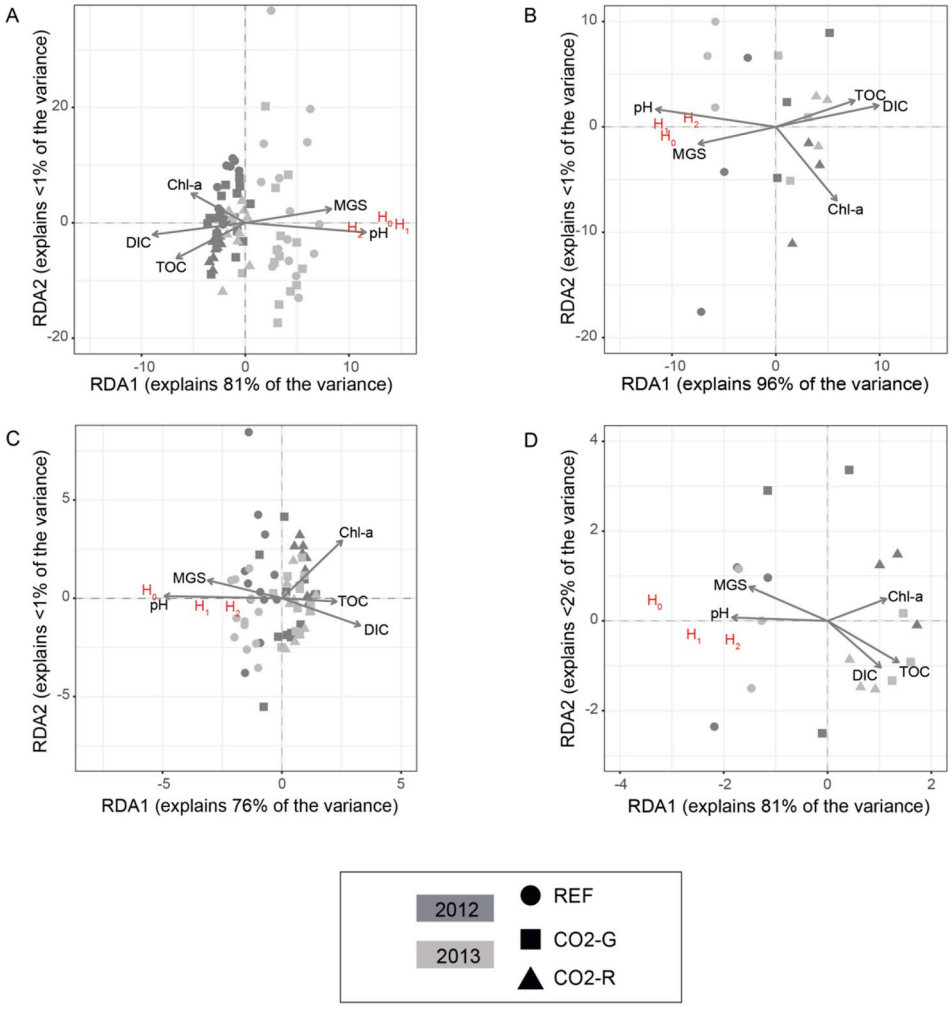

Fig. A.5. Redundancy analyses plots illustrating the contribution of environmental variables to explain the variance in OTUs / species diversity (Hills numbers) of Bacteria (ARISA; **A**: 0–10 cm, **B**: 0–2 cm) and Nematoda (**C**: 0–8 cm, **D**: 0–2 cm) at the three study sites (REF, CO2-G, CO2-R) based on abundance data of 2012 and 2013.

Table A.1
Sedimentary environmental data for the study locations sampled in 2012 and 2013, as presented in Molari et al. (2018). Data are presented as the average ± standard deviation (n = 3).

Year	Site	Layer	Silicate		Phosphate		Ammonium		Nitrite-Nitrate		pH_T		DIC		TA
			cm		µM		µM		µM		µM		mM		mM
2012	REF	0–2	7.6	± 2.7	0.5	± 0.4	1.2	± 0.4	1.6	± 1.1	7.5	± 0.1	2.5	± 0.1	2.5
		2–4	16.7	± 1.2	0.5	± 0.1	1.3	± 0.2	1.6	± 0.6	7.5	± 0.0	2.5	± 0.0	2.5
		4–6	21.8	± 3.4	0.5	± 0.1	2	± 2.2	0.6	± 0.5	7.4	± 0.1	2.4	± 0.0	2.5
		6–8	23.6	± 6.3	0.5	± 0.2	3l.7	± 2.0	0.4	± 0.5	7.4	± 0.1	2.5	± 0.2	2.5
		8–10	28.9	± 21.5	0.8	± 0.7	4.9	± 3.1	0.4	± 0.3	7.4	± 0.0	2.4	± 0.0	2.5
	CO2-G	0–2	131	± 163.9	3.7	± 5.7	2.3	± 3.9	0.4	± 0.3	5.8	± 0.3	19.5	± 18.5	8.8
		2–4	158.9	± 156.1	3.8	± 5.6	4	± 4.2	0.4	± 0.5	5.6	± 0.2	19.5	± 19.5	9.3
		4–6	163.3	± 149.5	5.6	± 8.5	5.8	± 5.9	0.5	± 0.7	5.6	± 0.2	26	± 16.2	9.7
		6–8	235.8	± 123.3	7.7	± 8.2	10.4	± 9.4	0.5	± 0.9	5.7	± 0.1	29.3	± 15.0	11.7
		8–10	275.4	± 108.8	7.9	± 8.0	11.2	± 9.9	0.3	± 0.5	5.6	± 0.1	31.4	± 13.6	12.8
	CO2-R	0–2	161.2	± 9.5	0.6	± 0.6	1.1	± 0.8	1.3	± 1.8	5.7	± 0.1	15.2	± 3.3	6.5
		2–4	370.8	± 148.8	0.3	± 0.1	3.5	± 2.0	1	± 1.2	5.7	± 0.1	24.1	± 6.7	9.4
		4–6	465.3	± 232.2	0.4	± 0.4	14	± 8.9	0.8	± 0.9	5.7	± 0.1	26	± 6.5	11
		6–8	505.3	± 224.1	1.3	± 1.7	14.8	± 9.6	1.1	± 1.4	5.7	± 0.1	29.6	± 7.6	10.5
		8–10	285.6	± 91.4	0.6	± 0.2	14.3	± 13.5	1.7	± 1.3	5.7	± 0.1	30.4	± 7.3	12.7
2013	REF	0–2	1.4	± 0.3	0	± 0.0	13.2	± 2.8	1.4	± 1.2	7.9	± 0.0	2.4	± 0.1	2.4
		2–4	3.6	± 1.1	0	± 0.0	12.2	± 5.0	4.1	± 2.4	7.7	± 0.1	2.5	± 0.1	2.4
		4–6	4.9	± 1.0	0	± 0.0	12.8	± 4.2	5.7	± 1.5	7.7	± 0.1	2.5	± 0.1	2.4
		6–8	7.2	± 2.7	0	± 0.0	12	± 3.7	7.7	± 3.4	7.7	± 0.2	2.5	± 0.1	2.3
		8–10	10.3	± 3.6	0	± 0.0	11.8	± 2.7	7.5	± 3.3	7.6	± 0.2	2.5	± 0.1	2.4

CO2-G	0–2	51.7	± 27.9	0.6	± 0.2	1.1	± 1.3	2	± 1.8	5.7	± 0.1	15.8	± 2.3	6.9
	2–4	69.8	± 31.9	0.7	± 0.4	3.1	± 0.3	2.5	± 0.9	5.6	± 0.0	19.6	± 2.3	7.9
	4–6	75.9	± 43.2	0.5	± 0.1	3.3	± 1.8	2.5	± 1.2	5.5	± 0.1	20	± 2.3	8
	6–8	67	± 16.9	0.5	± 0.1	3.4	± 2.0	2.6	± 1.3	5.5	± 0.2	20.4	± 2.6	8.3
	8–10	70.9	± 28.8	0.5	± 0.1	2.3	± 2.1	2.5	± 1.2	5.5	± 0.1	21.3	± 6.6	8.5
CO2-R	0–2	277.4	± 50.8	0	± 0.0	17	± 7.8	0.7	± 0.2	5.5	± 0.1	28.5	± 12.7	11.3
	2–4	301.3	± 115.6	0	± 0.0	19.2	± 9.9	0.7	± 0.2	5.6	± 0.1	33.5	± 7.2	13.4
	4–6	433.1	± 80.0	0.1	± 0.3	21	± 6.3	0.6	± 0.3	5.6	± 0.0	36	± 5.3	14.7
	6–8	350.2	± 165.5	0.8	± 1.0	15.5	± 1.9	0.5	± 0.2	5.6	± 0.1	36.2	± 6.1	14.4
	8–10	465.3	± 64.0	0.6	± 0.8	18.8	± 4.6	0.5	± 0.1	5.6	± 0.1	36.2	± 3.2	15.4

Year	TA	Iron		Manganese		Chl-a		TOC				Porosity	MGS	
	mM	μM		μM		μg/g		%				%	μm	
2012	± 0.1	0	± 0.0	0	± 0.0	1.52	± 0.57	0.027	± 0.002	49	± 2	856.6	± 52.8	
	± 0.2	0	± 0.0	0	± 0.0	1.45	± 0.52	0.024	± 0.003	51	± 2	849	± 26.2	
	± 0.1	0	± 0.0	0	± 0.0	1.1	± 0.36	0.023	± 0.006	50	± 2	828.5	± 57.6	
	± 0.0	0	± 0.0	0	± 0.0	1	± 0.22	0.023	± 0.006	51	± 1	807.6	± 15.3	
	± 0.1	0	± 0.0	0	± 0.0	1	± 0.33	0.021	± 0.003	50	± 3	811.7	± 61.6	
	± 8.5	591.5	± 894.7	23	± 39.9	3.35	± 0.85	0.046	± 0.024	52	± 4	783.6	± 92.3	
	± 9.0	466.4	± 680.5	21.6	± 33.0	2.25	± 0.42	0.03	± 0.010	51	± 5	709.4	± 127.3	
	± 7.4	427.1	± 510.6	23.7	± 29.2	1.47	± 0.22	0.033	± 0.004	53	± 4	772	± 135.7	
	± 6.9	600.1	± 503.1	30.7	± 24.9	1.54	± 0.43	0.035	± 0.002	50	± 3	715.6	± 85.0	
	± 5.5	736	± 581.2	36.8	± 22.4	1.27	± 0.61	0.032	± 0.010	49	± 1	677.2	± 50.4	
	± 0.1	65.8	± 87.2	14.2	± 1.3	13.42	± 4.03	0.056	± 0.008	52	± 5	503.6	± 0.2	
	± 1.8	335.1	± 240.3	28.1	± 8.0	10.89	± 1.48	0.052	± 0.008	52	± 4	507.7	± 29.1	
	± 2.0	556.8	± 516.6	32.1	± 6.0	9.31	± 2.51	0.047	± 0.006	54	± 3	519.5	± 33.7	
	± 1.4	613.9	± 369.6	34.2	± 6.6	6.18	± 2.40	0.058	± 0.006	53	± 2	508.4	± 63.0	
	± 2.2	741.1	± 407.2	39.6	± 8.6	4.57	± 1.99	0.051	± 0.007	46	± 1	532.6	± 55.0	
2013	± 0.1	1	± 1.1	0	± 0.0	1.8	± 0.32	0.042	± 0.003	36	± 3	686	± 42.0	
	± 0.1	0.8	± 0.7	0	± 0.0	1.22	± 0.11	0.037	± 0.004	42	± 2	686.8	± 65.1	
	± 0.1	0.6	± 0.3	0	± 0.0	1.01	± 0.20	0.049	± 0.004	58	± 23	702.3	± 70.0	
	± 0.1	0.6	± 0.3	0	± 0.0	0.71	± 0.27	0.041	± 0.004	43	± 1	705.9	± 3.1	
	± 0.2	0.6	± 0.7	0	± 0.0	0.59	± 0.27	0.038	± 0.012	37	± 9	711.5	± 76.7	
	± 0.4	99.5	± 41.9	9.9	± 8.0	1.73	± 0.35	0.087	± 0.013	42	± 14	636	± 63.8	
	± 0.7	212.2	± 138.9	18.8	± 3.4	1.4	± 0.07	0.063	± 0.017	42	± 9	618.6	± 60.8	
	± 0.6	233.1	± 143.4	19.6	± 3.0	1.17	± 0.09	0.053	± 0.011	41	± 4	661.5	± 65.6	
	± 0.4	219.9	± 111.7	19.4	± 0.9	1.17	± 0.15	0.047	± 0.008	42	± 2	635.3	± 101.5	
	± 2.1	268.8	± 125.1	21.8	± 5.8	0.98	± 0.38	0.061	± 0.010	44	± 3	646.4	± 82.2	
	± 4.2	487.6	± 509.3	30.1	± 11.0	5.78	± 0.96	0.097	± 0.021	37	± 6	574.9	± 41.9	
	± 3.1	657.2	± 396.6	40.6	± 11.7	4.65	± 0.21	0.066	± 0.013	43	± 2	553.2	± 57.8	
	± 2.0	773.8	± 222.2	47	± 14.3	4.04	± 0.43	0.055	± 0.011	42	± 2	526.2	± 58.6	
	± 2.5	764.9	± 177.3	44.7	± 7.8	2.77	± 0.24	0.059	± 0.002	43	± 8	553.8	± 42.7	
	± 0.7	813.3	± 71.6	43.6	± 5.6	2.57	± 0.78	0.065	± 0.019	39	± 4	529.4	± 28.0	

DIC: dissolved inorganic carbon; TA: total alkalinity; Chl-a: chlorophyll *a*; MGS: median grain size.

Table A.2
Alpha diversity for Bacteria based on 454-MPTS data considering the sediment layers 0–2 cm and 4–6 cm of sediment cores collected in 2012 (n = 3). Data are presented as the average ± standard deviation (n = 3).

Site	Layer	Total number of sequences	Chao1[1]		OTUs Alfa diversity						SSOabs[2]	Unique $OTU_{0.03}$[3]	Orders diversity[4]	
					H_0		H_1		H_2		%	%	n. orders	
REF	0–2 cm	6193 ± 1995	2551	± 141	1871	± 341	701	± 99	200	± 13	8	31	107	± 3
	4–6 cm	9258 ± 6398	2297	± 841	2159	± 1129	906	± 126	237	± 12	16	35	106	± 11
CO2-G	0–2 cm	11776 ± 8078	1100	± 157	1092	± 133	191	± 62	44	± 22	4	9	93	± 7
	4–6 cm	11698 ± 1203	1178	± 181	1219	± 106	164	± 54	47	± 8	5	10	93	± 3
CO2-R	0–2 cm	9739 ± 4810	958	± 368	964	± 474	171	± 23	43	± 4	4	8	88	± 14
	4–6 cm	7834 ± 2633	1011	± 173	935	± 205	140	± 9	42	± 5	2	7	85	± 5

[1] Calculated on subsampled 454-dataset (number sequences = 4346).
[2] Absolute singletons (SSO_{abs}) are OTUs consisting of sequences occurring only once in the full dataset (percentage relative to total OTUs of whole dataset).
[3] Unique OTUs are the dominant OTUs ($OTUs_{0.03}$ > 0.1%) present exclusively in one of three site investigated (percentage relative to total OTUs of whole dataset).
[4] Total number of bacterial orders.

Table A.3
Details of the statistical analyses on the Hill's numbers (H_0, H_1, H_2) from the natural sediments. Bacterial OTUs (ARISA) was analysed with three-factor ANOVA (model: Hill number ~ Site + Year*Site + Layer-nested-Site, data), Nematode species diversity with three-factor split-plot ANOVA with "sediment core" as random factor (model: Hill number ~ Site + Year*Site + Layer-nested-Site, random = ~1|Core, data), Polychaete species diversity with one-way ANOVA (model: Hill number ~ Site, data).

Size-class organisms	Hill numbers	Factors	Df	Sum Sq	Mean Sq	F value	P	Redundancy (%)
Bacteria	H_0	site	2	397590	198795	241.259	< 0.0001	63
		year	1	134	134	0.162	0.687	0
		site:year	2	393	196	0.238	0.788	0
		site:layer	3	411	137	0.166	0.919	0
	H_1	site	2	386367	193183	407.817	< 0.0001	73
		year	1	1475	1475	3.115	0.0787	0
		site:year	2	1490	745	1.573	0.2092	0
		site:layer	3	2397	799	1.687	0.17	0
	H_2	site	2	154090	77045	281.747	< 0.0001	64
		year	1	2319	2319	8.482	0.00387	1
		site:year	2	1676	838	3.064	0.04823	1
		site:layer	3	3712	1237	4.525	0.00406	0
Nematode	H_0	site	2	1984.6	992.3	139.972	< 0.0001	72
		year	1	5.63	5.63	0.7946	0.3828	0
		site:year	2	15.35	7.68	1.083	0.3568	1
		site:layer	3	102.54	34.18	4.8212	0.0039	4
	H_1	site	2	395.55	197.774	66.2195	< 0.0001	60
		year	1	1.52	1.519	0.5085	0.4836	0
		site:year	2	3.8	1.899	0.6359	0.5394	1
		site:layer	3	5	1.667	0.5581	0.6443	1
	H_2	site	2	175.782	87.891	39.2373	< 0.0001	47
		year	1	1.25	1.25	0.558	0.4633	0
		site:year	2	2.774	1.387	0.6193	0.5479	1
		site:layer	3	0.658	0.219	0.098	0.9609	0
Polychaete	H_0	site	2	43.364	21.6821	19.312	< 0.001	78
	H_1	site	2	25.0932	12.5466	18.603	< 0.001	77
	H_2	site	2	15.6196	7.8098	18.691	< 0.001	77

Df: degrees of freedom; Sum Sq: sum of the squares; Mean Sq: mean of the square; F value: statistic *F*; P: probability level; Redundancy (%): proportion of variance explained; site: REF, CO2-G and CO2-R; year: 2011, 2012 and 2013 for Bacteria and Nematode, and 2012 for Polychaete; layer: 0–2 cm and 2–10 cm for Bacteria, 0–2 cm and 2–8 cm for Nematode, and 0–5 cm for Polychaete.

Table A.4
Details of the statistical analyses on the Hill's numbers (H_0, H_1, H_2) from the transplant experiment. Bacteria (ARISA) and Nematode species diversity was analysed with three-factor split-plot ANOVA with "sediment core" as random factor (model: Hill number ~ Treatment + Layer-nested-Treatment, random = ~1|Core, data).

Size-class organisms	Hill numbers	Factors	Df	Sum Sq	Mean Sq	F value	P	Redundancy (%)
Bacteria	H_0	Treatment	3	7092.3	2364.1	41.077	< 0.0001	57
		Treatment:Layer	4	2816.1	704.02	12.233	< 0.0001	23
	H_1	Treatment	3	3059.6	1019.86	22.2783	0.0003	49
		Treatment:Layer	4	1201.1	300.28	6.5595	0.0003	19
	H_2	Treatment	3	959.46	319.82	5.5136	0.0239	24
		Treatment:Layer	4	533.51	133.38	2.2994	0.0738	13
Nematode	H_0	Treatment	3	302.648	100.883	15.9288	0.001	79
		Treatment:Layer	4	16.333	4.083	0.6447	0.6459	4
	H_1	Treatment	3	171.503	57.168	21.396	0.0004	78
		Treatment:Layer	4	21.398	5.35	2.0022	0.1872	10
	H_2	Treatment	3	90.895	30.2985	14.6966	0.0013	71
		Treatment:Layer	4	15.775	3.9437	1.9129	0.2017	12

Df: degrees of freedom; Sum Sq: sum of the squares; Mean Sq: mean of the square; F value: statistic *F*; P: probability level; Redundancy (%): proportion of variance explained; site: REF, CO2-G and CO2-R; treatment: within habitat transplants (REF/REF and CO2-R/CO2-R) and across habitats transplants (REF/CO2-R and CO2-R/REF); layer: 0–2 cm and 2–10 cm for Bacteria, 0–2 cm and 2–4 cm for Nematode.

Table A.5
Output of standard and partial redundancy analyses (RDAs). Significant variables used for variation portioning analysis and partial RDA are in bold.

0–10 cm Bacteria (n = 90)					
RDA: Hill Numbers ~ pH + Chl.a + TOC + MGS + Silicate	adjR^2	0.804			
	ANOVA	Df	SS	F	P
	pH	**1**	**3582.2**	**346.7**	**0.001**
	Chl.a	1	22.5	2.2	0.153
	TOC	1	22.6	2.2	0.148

	MGS	**1**	**80**	**7.7**	**0.004**
	Silicate	**1**	**119.9**	**11.6**	**0.001**
	Residual	84	867.8		
pRDA: Hill Numbers ~ pH + Condition (MGS,Silicate)	adjR^2	0.401			
	ANOVA	Df	SS	F	P
	pH	1	1874.1	163.4	0.001
	Residual	87	997.8		
pRDA: Hill Numbers ~ G.size + Condition (pH + Silicate)	adjR^2	0.003			
	ANOVA	Df	SS	F	P
	MGS	1	25.1	2.4	0.104
	Residual	86	888.8		
pRDA: Hill Numbers ~ Silicate + Condition (pH + MGS)	adjR^2	0.022			
	ANOVA	Df	SS	F	P
	Silicate	1	109.0	10.5	0.002
	Residual	86	888.8		
0–2 cm Bacteria (n = 18)					
RDA: Hill Numbers ~ pH + Chl.a+TOC + MGS + Silicate	adjR^2	0.937			
	ANOVA	Df	SS	F	P
	pH	**1**	**4979.1**	**236.5**	**0.001**
	Chl.a	1	24.7	1.2	0.313
	TOC	1	7	0.3	0.591
	MGS	**1**	**109.4**	**5.2**	**0.042**
	Silicate	**1**	**287.2**	**13.6**	**0.007**
	Residual	12	252.6		
pRDA: Hill Numbers ~ pH + Condition (MGS,Silicate)	adjR^2	0.55			
	ANOVA	Df	SS	F	P
	pH	1	2969.5	72.4	0.001
	Residual	15	615.5		
pRDA: Hill Numbers ~ G.size + Condition (pH + Silicate)	adjR^2	0.005			
	ANOVA	Df	SS	F	P
	MGS	1	65.5	1.6	0.228
	Residual	15	615.5		
pRDA: Hill Numbers ~ Silicate + Condition (pH + MGS)	adjR^2	0.056			
	ANOVA	Df	SS	F	P
	Silic	1	322.5	13.5	0.001
	Residual	15	358.5		

0–10 cm Bacteria (n = 90)	0–8 cm Nematoda (n = 72)					
RDA: Hill Numbers ~ pH + Chl.a+TOC + MGS + Silicate	RDA: Hill Numbers ~ pH + Chl.a+TOC + MGS + Silicate	adjR^2	0.751			
		ANOVA	Df	SS	F	P
		pH	**1**	**37.9**	**210.0**	**0.001**
		Chl.a	**1**	**0.9**	**4.8**	**0.026**
		TOC	1	0.1	0.6	0.439
		MGS	1	0.4	2.0	0.142
		Silicate	1	0.2	1.2	0.285
		Residual	66	11.9		
pRDA: Hill Numbers ~ pH + Condition (MGS,Silicate)	pRDA: Hill Numbers ~ pH + Condition (Chl.a)	adjR^2	0.561			
		ANOVA	Df	SS	F	P
		pH	1	28.6	156.3	0.001
		Residual	69	12.6		
pRDA: Hill Numbers ~ G.size + Condition (pH + Silicate)	pRDA: Hill Numbers ~ Chl.a+Condition (pH)	adjR^2	0.013			
		ANOVA	Df	SS	F	P
		Chl.a	1	0.9	4.7	0.022
		Residual	69	12.6		
pRDA: Hill Numbers ~ Silicate + Condition (pH + MGS)						
0–2 cm Bacteria (n = 18)	0–2 cm Nematoda (n = 18)					
RDA: Hill Numbers ~ pH + Chl.a+TOC + MGS + Silicate	RDA: Hill Numbers ~ pH + Chl.a+TOC + MGS + Silicate	adjR^2	0.755			
		ANOVA	Df	SS	F	P
		pH	**1**	**26.9**	**47.6**	**0.001**
		Chl.a	1	0.9	1.6	0.233
		TOC	1	0.6	1.1	0.315
		MGS	***1***	***1.8***	***3.1***	***0.095***
		Silicate	***1***	***2.3***	***4.1***	***0.058***
		Residual	12	6.8		
pRDA: Hill Numbers ~ pH + Condition (MGS,Silicate)	pRDA: Hill Numbers ~ pH + Condition (MGS,Silicate)	adjR^2	0.376			
		ANOVA	Df	SS	F	P
		pH	1	13.6	26.4	0.001
		Residual	14	7.2		
pRDA: Hill Numbers ~ G.size + Condition (pH + Silicate)	pRDA: Hill Numbers ~ MGS + Condition (pH + Silicate)	adjR^2	0.108			

		ANOVA	Df	SS	F	P
		MGS	1	4.3	8.3	0.005
		Residual	14	7.2		
pRDA: Hill Numbers ~ Silicate + Condition (pH + MGS)	pRDA: Hill Numbers ~ Silicate + Condition (pH + MGS)	adjR^2	0.051			
		ANOVA	Df	SS	F	P
		Silicate	1	2.3	4.5	0.047
		Residual	14	7.2		

n: number of observations; Hill number: H_0, H_1, H_2; Chl.a: chlorophyll a; TOC: total organic carbon; MGS: mean grain size; adjR^2: adjusted R^2; Df: degrees of freedom; SS: sum of the squares; F: statistic F; P: probability level; RDA: standard redundancy analysis; pRDA: partial redundancy analysis.

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